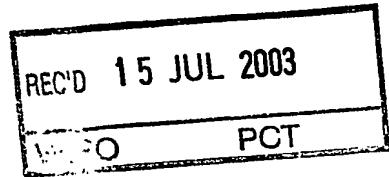
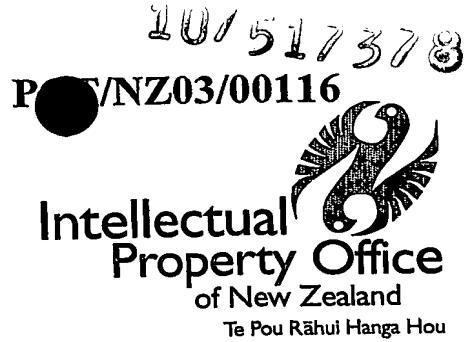


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CERTIFICATE

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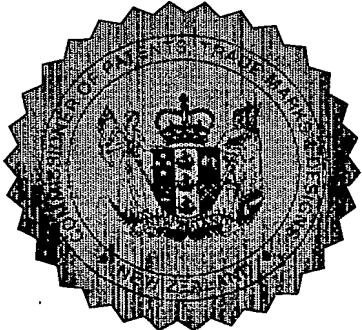
I hereby certify that annexed is a true copy of the Provisional Specification as filed on 10 June 2002 with an application for Letters Patent number 519456 made by Wool Research Organisation of New Zealand (Inc.)

I further certify that pursuant to a claim under Section 24(1) of the Patents Act 1953, a direction was given that the application proceed in the name of Wool Research Organisation of New Zealand (Inc.) and UNIVERSITY OF OTAGO.

Dated 30 June 2003.

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Patents Form No. 4

Our Ref: RL802458

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PROVISIONAL SPECIFICATION

Orthopaedic Materials Derived from Keratin

We, **Wool Research Organisation of New Zealand (Inc.)**, An Incorporated Society, of Cnr Springs Road & Gerald Street, Lincoln, Canterbury New Zealand do hereby declare this invention to be described in the following statement:

PT0409855

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ORTHOPAEDIC MATERIALS DERIVED FROM KERATIN

Field of the Invention

This invention relates to the preparation of medical materials from hard alpha keratins derived
5 from animal sources such as wool, hair, horns, hooves, and scales. The keratin materials described
are biocompatible, biointegratable, and biodegradable and the primary application of the materials
is in orthopaedic surgery for replacement and augmentation of bone, and fixation and
immobilization of bone fractures.

10 Background to the Invention

Keratins are a class of structural proteins widely represented in biological structures, especially in
epithelial tissues of higher vertebrates. Keratins may be divided into two major classes, the soft
keratins (occurring in skin and a few other tissues) and the hard keratins (forming the material of
nails, claws, hair, horn, feathers and scales).

15

The toughness and insolubility of hard keratins, which allow them to perform a fundamental
structural role in many biological systems, are the desirable characteristics found in many of the
industrial and consumer materials derived from synthetic polymers. In addition to possessing
excellent physical properties, keratin, as a protein, is a polymer with a high degree of chemical
20 functionality and consequently exhibits many properties that synthetic polymers cannot achieve.

Keratin is therefore, well suited to the development of medical products with high-value, niche
market applications. Medical materials which are absorbed (resorbed) by the body tissues after
fulfilling their function are an example of an area of high value products in which the specific
characteristics of keratin allow it to outperform both natural and synthetic competitive materials.

25

Many tissues of the body including bone are continually renewed. New bone matrix (which will
become mineralized) is laid down principally by specific cells called osteoblasts, and the different

components of bone are removed by osteoclasts. An implanted material which is removed and replaced with bone tissue by this biological process will have a greater advantage over those materials which break down by other mechanisms within the body e.g. chemical degradation. It is desirable that new bone is formed juxtaposed to the surface of the implanted material, thereby integrating this material into the tissue until it is completely resorbed and replaced.

Bone may be categorized into four microstructural components: cells, organic matrix, inorganic matrix, and soluble signalling factors. Osteoblasts are metabolically active secretory cells that express soluble signaling factors and osteoid, a product whose extracellular modification yields an organic insoluble substratum consisting mostly of type I collagen. Expression of these products by osteoblasts occurs during maintenance (e.g. remodelling), and repair of bone. Monocyte-macrophage precursors found in the bone marrow enter the circulation, and through asynchronous fusion produce a multinucleated cell up to 100 microns in diameter with an average of 10 to 12 nuclei, known as an osteoclast. Osteoclasts have a ruffled border and this constitutes the resorptive territory of the osteoclast where enzymatic breakdown of the bone surface occurs. The term 'remodelling' is used to describe the dynamic events associated with bone repair and homeostasis in the mature individual. The sum of the processes associated with homeostatic remodelling is known as activation-resorption-formation. Osteoblasts are activated by signalling factors and vacate an area of bone; osteoclasts become stimulated, home in to the osteoblast-vacant zone, attach, resorb, and, in response to an as yet unidentified signal, cease resorbing and abandon their attachment. Osteoclastic resorptive pits become repopulated by a contingent of osteoblasts that express osteoid, which calcifies, restoring bone. In humans, the activation-resorption-formation processes take between 3 and 6 months.

Following an insult to bone (e.g. fracture or surgical removal of a tumor) there is extensive bleeding and in 2 to 5 days the haemorrhage forms a large blood clot. Neovascularization begins to occur peripheral to this blood clot. There is also the standard inflammatory response occurring

in the surrounding soft tissues leading to polymorphonuclear leucocytes, macrophages, and mononuclear cells accumulating in the periphery of the clot. By the end of the first week, most of the clot is organised by invasion of blood vessels and early fibrosis. The earliest bone (woven bone) is formed after 7 days. Since bone formation requires a good blood supply, the woven bone 5 spicules begin to form at the periphery of the clot where vascularisation is greatest. Pluripotential mesenchymal cells from the surrounding soft tissues and from within the bone marrow give rise to osteoblasts that synthesize the woven bone. Frequently cartilage is also formed and eventually is replaced by endochondral ossification. The granulation tissue containing bone-cartilage is termed a callus [Inflammatory phase].

10

After the first week, the next stage begins and extends for several months, depending upon the degree of movement and fixation. By this stage, the acute inflammatory cells have dissipated and the reparative process involving the differentiation of pluripotential cells into fibroblasts and osteoblasts commences. Repair proceeds from the periphery towards the centre and accomplishes 15 two objectives: one, it organises and resorbs the blood clot; and two, more importantly, it furnishes neovascularisation for the construction of the callus, which eventually bridges the bone-deficient site. The events leading to the repair are as follows. Large numbers of osteoclasts from the surrounding bone move into the healing site. New blood vessels accompany these cells supplying nutrients and providing more pluripotential cells for cell renewal. The site is remodelled by 20 osteoclasts [Reparative phase].

In several weeks the callus has sealed the bone ends and remodelling begins, in which the bone is reorganised so that the original cortex is restored [Remodelling phase].

25 Objects of the Invention

An object of the invention is to provide an orthopaedic material derived from keratin and a method of using such materials which has a wide variety of uses and applications which offer useful

alternatives to medical practitioners.

Further objects and advantages of the invention will become apparent from the following descriptions which are given by way of example.

5

Summary of the Invention

In its broadest aspect the invention provides a biocompatible material derived from keratin and methods of forming this material into a range of products for orthopaedic use.

- 10 The biocompatible material is a highly s-sulfonated form of keratin enriched in intermediate filament proteins of high molecular weight, preferentially isolated from a keratin source such as wool, and separated from other classes of keratin protein, using methods such as those outlined in N.Z. Patent specification No. 512725.

- 15 One aspect of the invention is a biocompatible material in the form of a highly porous keratin enriched in intermediate filament protein, for use as a bone replacement and augmentation product. The product can be used for example to replace bone lost due to clinical conditions such as tumors or trauma, and will promote healing by acting as a scaffold for the laying down of new bone. The porous keratin scaffold is resorbed and replaced by new woven bone which will subsequently be
20 remodelled into cancellous and cortical bone.

The method of reforming the s-sulfonated keratin enriched in intermediate filament protein into a porous matrix involves the following steps:

- 25 compressing the protein in the presence of a soluble porogen;
- 25 washing of the protein material;
- applying a subsequent chemical treatment to remove the porogen and strengthen the material;

freeze drying of the protein material to thereby create a highly porous network.

Through controlling the amount and nature of the soluble porogen, the pore sizes are selected to allow the infiltration of osteoprogenitor cells to facilitate the colonization of the implanted keratin
5 matrix leading to degradation of the keratin matrix and replacement by new bone.

A further aspect of the invention is a biocompatible material in the form of a tough, dense keratin material enriched in intermediate filament protein, which can be used as a product for bone fixation and immobilization. For example plates, pins and screws manufactured from this
10 biocompatible keratin material can be used for the treatment of fractures by internal fixation.

As mentioned above, a degradable keratin appliance will provide enough flexibility to stimulate new bone growth, unlike some rigid permanent materials currently used, as well as gradually transferring the load to the healing bone. Furthermore subsequent surgery is not needed to remove
15 the keratin-derived fixation appliances or devices.

The method of reforming the s-sulfonated keratin enriched in intermediate filament protein into a tough, dense material for use as an internal fixation appliance in the treatment of bone fractures involves compressing the biocompatible protein in the presence of moisture, chemicals, and in
20 some cases heat, to form a desired shape. The formation of crosslinks within the material to ensure strength and toughness under biological conditions occurs during compression, or subsequently through chemical treatment.

A further aspect of the invention is the controlled use of reducing agents to remove the sulfonate group from the s-sulfonated keratin and reform the disulfides originally present in the native keratin. This serves the dual purpose of firstly, providing strength and toughness under biological
25 conditions by effectively polymerizing the keratin protein through an extended network of

disulfide crosslinks; and secondly, controlling the rate and extent of biodegradation that occurs by impeding enzymatic digestion of the material. By controlling the rate of biodegradation, the invention allows the keratin products to be used in applications where a range of healing times are desirable.

5

The reconstituted keratin described in this patent specification is completely biocompatible and does not elicit any significant foreign body type immune reaction. The keratin is degraded by the normal processes occurring in bone described above and is replaced with normal bone. Therefore in bone replacement and augmentation, this keratin has similar properties to autologous bone. In 10 the case of treatment of fractures, appliances made of this keratin will have the necessary physical properties to carry out the fixation and immobilization of the bone segments, and once this function is fulfilled the keratin will gradually resorb and eventually disappear from the tissues. During the initial period of resorption, the gradually weakening keratin appliance will stimulate new bone formation by subjecting the bone to increasing functional loads. This will prevent a 15 stress shielding effect which is found with metal appliances. Furthermore the degradation and resorption of keratin is a great advantage compared to metal appliances which are either permanently retained in the body or require a second surgical procedure to remove them.

Description of Preferred Embodiments of the Invention

20 Highly s-sulfonated keratin, derived from an animal source such as wool, hair, horns or hooves, when purified to isolate the intermediate filament protein component, is a biocompatible/biodegradable material ideal for use in a range of products for orthopaedic use. The keratin may be prepared by methods such as those outlined in New Zealand Patent specification No. 512725. The material may be reformed into different matrices for use as products in 25 orthopaedic care. Some of these matrices retain the sulfonate functionality originally present in the keratin material, whereas others are chemically treated as part of a reconstitution process to remove the sulfonate functionality and restore the disulfide bonds originally present within the

keratin.

In one embodiment of the invention the s-sulfonated keratin enriched in intermediate filament protein is reconstituted into a highly porous matrix for use as a bone augmentation product. This is
5 achieved by compressing the keratin protein in the presence of water and a soluble porogen, such as sodium chloride. In one example, 0.5g of s-sulfonated keratin intermediate filament protein powder, ground to a particle size of 125-200 micron, is mixed with 0.5ml of water and 0.03g of sodium chloride. This mixture is packed into a 12mm diameter die and pressed to a pressure of 150kPa for 2 minutes. On removal from the die a 3mm diameter cylinder is cut from the pellet and
10 the cylinder is soaked in a chemical treatment solution, in order to wash out the porogen and leave a porous matrix, while also removing the sulfonate functionality from the keratin and restoring disulfide crosslinks to the protein. This chemical treatment may take one of two forms. Firstly, a solution of 0.25M ammonium thioglycollate containing 0.1M sodium phosphate buffered to pH 7.0 can be used to treat the cylinder for a period of 18 hours. Residual chemicals are removed by
15 subsequent washing of the pellet in water successively for 10, 40 and 10 minute periods. After washing the cylinder is then freeze dried. Alternatively, the cylinder can be subjected to chemical treatment in a solution containing 0.1M thioglycollic acid for 18 hours. Following a brief wash in water, residual chemicals are removed by washing the cylinder in a solution of 0.1M TRIS 11.25 mM calcium chloride for 72 hours. The solution is refreshed every 24 hours. Following a further
20 wash in water the cylinder is freeze dried.

The pore size in the matrix can be controlled by varying the quantity of sodium chloride used in the preparation. For example, typical pore size for a preparation using 0.06g of sodium chloride per gram of protein is 50-150 microns, whereas 0.14g of sodium chloride per gram of protein
25 results in pores of up to 320 microns.

The biological properties of the porous keratin matrix prepared using the thioglycollic acid method

are demonstrated both in vitro and in vivo. In vitro, the material is not cytotoxic and supports the growth of human and sheep fibroblasts. Direct contact of the porous matrix with cells, as described in ISO 10993-5, using sheep fibroblasts produced the following effects. Wells of polystyrene cell culture plates containing either the porous matrix or no matrix as a control were initially seeded with ~10,000 cells (0 hours). During the first 24h post-seeding, the cultures experienced a lag time as evidenced by a decline in cell numbers. This phenomenon has been recognised in all assays performed and the drop is observed in control wells in addition to those containing the test materials. Experimentation has shown that this lag time lasts for less than 12h and that the exponential phase of growth begins at this point. Population doublings occur approximately every 5 24h-48h with subconfluence (approximately 80% confluence) marking the end of logarithmic growth. This corresponds to the end of the experimental time course (5 days or 120h). Extended time-course experiments have indicated a plateau in cell growth shortly after this with full confluence of the culture. Contact inhibition and depletion of nutrients play a key role in limiting the growth rate at this point and the monolayer culture exhibits signs of cell death (i.e. loss of 10 membrane integrity, reduction in cell numbers, vacuolisation of individual cells). During the assay, cells were witnessed to attach to the upper surface of the disks. By light microscopy, the morphological appearance of these cells was deemed similar on all substrates compared to the no-matrix control. Similar assays with human fibroblasts produced very similar results, with typical 15 fibroblast growth curves occurring in the presence of the porous matrix and approximately 80% confluence reached after 120 hours in culture. The control wells had reached 100% confluence at 20 this time.

The in vivo study involved the keratin material treated by the above method, and also a composite of this material containing 6% hydroxyapatite, and manufactured as rods 3 mm diameter, 3 mm 25 length and sterilized by gamma radiation (2.8 Mrads) being implanted into the midshaft (cortical bone) and proximal and distal ends (cortico-cancellous bone) of the long bones of the hind limbs of adult sheep. The tissue responses to the implanted material were studied by histological

examination of biopsy samples at 10 days, 3, 6, 8, 12 and 24 weeks. The bone tissue response to the material prepared using thioglyciolic acid showed minimal foreign body type immune reaction to the presence of the implant and there was only a thin layer of granulation tissue (leading to fibrosis) formed between the implant and the surrounding bone. Within 3 to 6 weeks the implant material was colonized by osteoid tissue leading to the laying down of woven bone in the spaces created by resorption of the keratin implant material, and the new bone was joined to the surrounding bone at around 6 to 8 weeks. From 6 weeks onwards remodelling of woven bone into corticocancellous bone occurred. The continuation of this process led to complete integration of the implant material which was replaced by mature bone, and the bone defect was completely healed.

Furthermore the physical properties of this porous material were investigated by manufacturing it in the form of plates 12 mm length, 4 mm width, 3 mm depth and implanted subcutaneously in the sheep. The data obtained by testing the plates on an Instron machine showed that the tensile properties weakened by approximately 10% over a period of 3 to 6 weeks. This was consistent with a loss in dry weight of about 10% at this time. These findings support the ability of this material to stimulate new bone formation and prevent stress shielding, and gradual resorption of this material.

In a further embodiment of the invention the s-sulfonated keratin enriched in intermediate filament protein is reconstituted into a tough, dense material for use as a bone fixation product. This is achieved by compressing the keratin protein in the presence of water. In one example, 0.5g of s-sulfonated keratin intermediate filament protein powder, ground to a particle size of 125-200 micron, is mixed with 0.5ml of water. This mixture is packed into a 12 mm diameter die and pressed to a pressure of 150 kPa for 2 minutes. On removal from the die a desirable shape is cut from the pellet, such as a 12mm by 4 mm block. The block is soaked in a chemical treatment solution, in order to remove the sulfonate functionality from the keratin and restore disulfide

crosslinks to the protein. This chemical treatment may take one of two forms. Firstly, a solution of 0.25M ammonium thioglycollate containing 0.1M sodium phosphate buffered to pH 7.0 can be used to treat the block for a period of 18 hours. Residual chemicals are removed by subsequent washing of the block in water successively for 10, 40 and 10 minute periods. After washing the
5 block is then allowed to dry in air at room temperature. Alternatively, the block can be subjected to chemical treatment in a solution containing 0.1M thioglycollic acid for 18 hours. Following a brief wash in water, residual chemicals are removed by washing the block in a solution of 0.1M TRIS 11.25mM calcium chloride for 48 hours. The solution is refreshed every 24 hours. Following a further wash in water the block is dried in air at ambient temperature.

10

The biological properties of the dense keratin material were demonstrated in vitro and the biophysical properties were examined in vivo. In vitro, the material is not cytotoxic and supports the growth of human and sheep fibroblasts. In a similar manner to that outlined above for the porous matrices, direct contact of the porous matrix with cells, as described in ISO 10993-5, using
15 sheep fibroblasts produced the following effects. Wells of polystyrene cell culture plates containing either the non-porous matrix or no matrix as a control were initially seeded with ~10,000 cells (0 hours). During the first 24h post-seeding, the cultures experienced a lag time as evidenced by a decline in cell numbers. This phenomenon has been recognised in all assays performed and the drop is observed in control wells in addition to those containing the test
20 materials. Experimentation has shown that this lag time lasts for less than 12h and that the exponential phase of growth begins at this point. Population doublings occur approximately every 24h-48h with subconfluence (approximately 80% confluency) marking the end of logarithmic growth. This corresponds to the end of the experimental time course (5 days or 120h). Extended time-course experiments have indicated a plateau in cell growth shortly after this with full
25 confluence of the culture. Contact inhibition and depletion of nutrients play a key role in limiting the growth rate at this point and the monolayer culture exhibits signs of cell death (i.e. loss of membrane integrity, reduction in cell numbers, vacuolisation of individual cells). During the assay,

cells were witnessed to attach to the upper surface of the disks. By light microscopy, the morphological appearance of these cells was deemed similar on all substrates compared to the no-matrix control. Similar assays with human fibroblasts produced very similar results, with typical fibroblast growth curves occurring in the presence of the porous matrix and approximately 80% confluence reached after 120 hours in culture. The control wells had reached 100% confluency at this time.

The biophysical properties (modulus of elasticity, modulus of rupture, tensile strength) were tested by implanting the material treated with thioglycollic acid and manufactured as plates 12 mm length, 4 mm width, 3 mm depth subcutaneously in adult rats. The plates were removed from the rats at 1, 3, 6 and 12 weeks and the physical strengths evaluated. The modulus of elasticity showed a decrease of 40 to 70% over a period of 3 to 6 weeks. The loss in dry weight at these two times was 5 to 10% which was in agreement with our study in the sheep with plates of the porous material.

An aspect of the invention is the use of reducing agents, such as ammonium thioglycollate or thioglycollic acid described above, in order to remove the sulfonate functionality from the protein and restore the disulfide bonding originally present in the native keratin. In the sulfonate form the keratin is soluble above pH 4 and rapidly resorbed in vivo. In order to sustain the material for a longer time within the body, and control the rate of degradation and resorption of the material, reductive agents that remove the sulfonate function and crosslink the protein can be employed. The extent to which reductive agents are used, the time of exposure and concentration of reagents, affects the ratio of sulfonate groups to disulfide bonds present within the material. This in turn affects the strength and rate of degradation in vivo. Other crosslinking agents, such as those employed to modify the properties of other biological biomaterials, for example glutaraldehyde and ethyldimethylaminopropylcarbodiimide hydrochloride (EDC) which are used to modify the properties of collagen biomaterials, can also be used to modify the properties of the keratin

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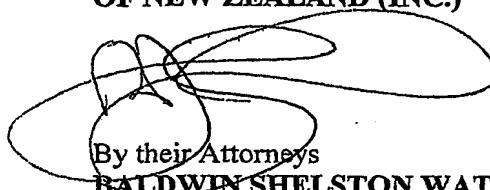
biomaterials.

Particular examples of the invention have been described and it is envisaged that improvements and modifications can take place without departing from the scope thereof.

5

WOOL RESEARCH ORGANISATION
OF NEW ZEALAND (INC.)

10



By their Attorneys

BALDWIN SHELSTON WATERS

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